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Nitrilase Catalyzes Amide Hydrolysis as Well as Nitrile Hydrolysis

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While amides were reported to be completely inert as substrates for all nitrilases reported to date, the nitrilase from *Rhodococcus rhodochrous* J1, which catalyzes the hydrolytic cleavage of the C-N triple bond in nitrile to form acid and ammonium, was surprisingly found to catalyze hydrolysis of amide to acid and ammonium stoichiometrically. This nitrilase exhibited a K_m of 2.94 mM for benzamide, similar to that for benzonitrile as the original substrate (2.10 mM), but the V_{max} for benzamide was six orders of magnitude lower than that for benzonitrile. Benzamide inhibited the nitrilase reaction in a reversible, apparently competitive manner. A mutant nitrilase containing alanine or serine instead of Cys165, which is essential for nitrilase catalytic activity, showed no amidase activity. This observation demonstrated that Cys165 plays a crucial role in the hydrolysis of amides as well as nitriles. Together with some reports that certain nitrilases were previously noted to produce low amounts of amide as a by-product from nitrile, the above unexpected findings suggested the existence of a common tetrahedral intermediate in the nitrilase reaction involving nitrile or amide as a substrate.

Nitriles are organic compounds containing a carbon-nitrogen triple bond and occur in nature; cyanoglycosides and cyanolipids are formed by a wide range of plants [1]. On the other hand, nitriles such as acetonitrile, adiponitrile, and acrylonitrile are widely manufactured by the chemical industry. Nitriles are very toxic and are generally bioundegradable compounds. However, some microorganisms can utilize nitriles as carbon and/or nitrogen sources [2–4]. The microbial degradation of nitriles proceeds through two distinct enzymatic pathways [4–8]; nitrilase [9–11] catalyzes the direct hydrolysis of a nitrile to the corresponding carboxylic acid plus ammonium, whereas nitrile hydratase [6,12–14] catalyzes the hydration of a nitrile to the corresponding amide, followed by its conversion to an acid plus ammonium by amidase [15,16].

Nitrilase has versatile functions in both eukaryotic and prokaryotic forms [9]. This enzyme is involved in the biosynthesis of the plant hormone, indole-3-acetic acid, from indole-3-acetonitrile [3,17–20]. Nitrilase is also expected to be a useful biocatalyst for organic chemical processing [9,21,22], because this environmentally friendly bioconversion allows clean and mild syntheses with high selectivity and yields. Very recently, surprisingly, nitrilase genes were even discovered in human, flies, and worms [23]. We initially identified the unique catalytically active cysteine residue conserved in three nitrilases [2,9,21,24]. Based upon our experimental data, Bork and Koonin proposed a novel C-N hydrolase superfamily [25] consisting of nitrilase, cyanide hydratase, aliphatic amidase and β-ureidopropionase (= β-alanine synthase; also called *N*-carbamoyl-β-alanine amidohydrolase). It is noteworthy that nitrilase shows significant sequence similarity to aliphatic amidase from *Pseudomonas aeruginosa*. Aliphatic amidase [26,27] also has an essential cysteine residue as the active site nucleophile of the catalytic mechanism [28].

We are interested in how C-N hydrolases evolved. Both amide and nitrile contain a C-N bond, although the numbers of covalent bonds between the carbon and nitrogen atoms are different: $CO\text{-}NH_2$ in the former and C ß N in the latter. Since no nitrilases identified to date have been reported to act on amides, we examined the amidase activity of the *Rhodococcus rhodochrous* J1 nitrilase. Here, we describe unusual substrate specificity characteristics of the nitrilase in that this enzyme catalyzes the hydrolysis of amides to acids and ammonia at the same catalytic site as used in the nitrile-hydrolysis reaction. The unique catalytic mechanism of this process is also discussed.

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MATERIALS AND METHODS

Enzyme preparation. Nitrilase was purified from cells of *Escherichia coli* JM109 harboring pNJ20, which carried the *R. rhodochrous* J1 nitrilase gene [24], using the following modification of the previously reported method [24] in order to obtain a highly purified enzyme. Cell-free extracts prepared from the cells (wet mass, 41 g) were fractionated with ammonium sulfate (25-50%, mass/vol.), followed by dialysis against 0.01 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol, which was used as a standard buffer throughout purification, unless otherwise specified. The dialyzed enzyme solution was applied to a DEAE-Sephacel column (Pharmacia, Sweden) equilibrated with the 0.01 M buffer containing 10% (mass/vol.) glycerol. After the column had been washed fully with the buffer containing 10% glycerol and 0.2 M KCl, the enzyme was isocratically eluted with the buffer containing 10% glycerol and 0.25 M KCl. The rate of sample loading and column elution was kept at 30 ml/h. The active fractions were combined and precipitated with ammonium sulfate at 80% saturation. The resultant precipitate was centrifuged and dissolved in the 0.1 M buffer. The enzyme suspension was dialyzed against the 0.01 M buffer, and then the dialyzed enzyme was applied to a Cellulofine GCL-2000 superfine column (Seikagaku Kogyo Co., Tokyo) and eluted with the 0.01 M buffer. Active fractions were precipitated with ammonium sulfate at 60% saturation and then dialyzed against the 0.1 M buffer.

With the exception of the amount of cells, mutant nitrilases were purified as described above from *E. coli* JM109 carrying pNJ20-165S (wet cell mass, 9 g) or pNJ20-165A (wet cell mass, 10.2 g), in which the codons corresponding to the active cysteine-165 of the nitrilase were replaced with those encoding serine or alanine, respectively [24].

Enzyme assay. The nitrilase activity of the purified nitrilase was assayed by the method described previously [29]. Nitrilase activity was defined as the amount of enzyme that catalyzed the formation of benzoic acid from benzonitrile as the substrate under the above conditions.

Velocity of nitrilase-catalyzed amidase activity was expressed as μmol benzoic acid formed $min^{-1} \cdot$ mg $protein^{-1}$ from benzamide.

Analytical methods for nitrilase. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) was performed by the method of Laemmli [30]. Proteins were stained with either Coomassie brilliant blue R-250 or silver according to Oakley *et al.* [31].

Anti-(amidase) antiserum prepared previously [16], was used in Western blot experiments. Immunoblotting of SDS/PAGE gels was performed according to Kyse-Andersen [32].

RESULTS AND DISCUSSION

The *R. rhodochrous* J1 nitrilase was highly purified from the *E. coli* transformant containing only the enzyme gene, as described under Materials and Methods. Neither gels stained with Coomassie brilliant blue nor with silver after SDS/PAGE showed protein bands other than the nitrilase band. No immunoreactive bands were detected (data not shown) in the Western blots for the above SDS/PAGE with the anti-(amidase) antiserum which was previously reported to recognize the *R. rhodochrous* J1 amidase [16]. The physicochemical properties (*e.g.*, molecular mass, optimum temperature and pH) of the nitrilase purified from the *E. coli* transformant were in agreement with those of the nitrilase purified from *R. rhodochrous* J1 [29]; these findings are also reported previously [24]. These data strongly demonstrated that the nitrilase prepared here had very high purity and did not contain any other contaminant proteins exhibiting amidase activity. The purity of benzamide (Aldrich Chemicals Company, Milwaukee, USA), which was used as the substrate in the following experiments, was also very high (*i.e.*, > 99.9%).

Previous experiments [29] demonstrated that benzamide is not degraded by the *R. rhodochrous* J1 nitrilase. The assay condition, in which the reaction (3 ml) was performed for 15 min using the purified enzyme (1.2 pmol; this value was calculated based on the 78 kDa molecular mass in the dimer subunit form of this enzyme) and the sensitivity in the detection by high-performance liquid chromatography was 16-fold less than that used for the assay described below, is likely to be the reason for the detection limit in the benzoic acid determination. When a large amount of nitrilase was used, however, amidase activity was observed. The reaction was carried out at 25°C using the purified nitrilase (178 nmol) in the standard reaction mixture (3 ml) for nitrilase as described under Materials and Methods, with the exception of the replacement of benzonitrile with benzamide as the substrate. Aliquots (0.1 ml) of the reaction mixture were taken at various time points (*i.e.*, 0.5, 1, 2, and 3 h), and the reaction was terminated by adding 10 μl of 1 M HCl. The disappearance of benzamide corresponded closely to the appearance of benzoic acid. With the nitrilase, 1 mol of benzoic acid and ammonium were formed per mole of benzamide degraded. The formation of no other products was detected. The activity of the nitrilase toward benzamide was 3.34×10^{-5} μmols/min/mg of protein, while that toward benzonitrile was 15.3 μmols/min/mg of protein [29]; relative activity in the former case was 0.00022% when the activity in the latter case was taken as 100%. Although the catalytic activity toward benzonitrile was 6 orders of magnitude larger than that toward benzamide, the amidase activity was definitely one of the properties of this nitrilase.

The V_{max} for benzamide was 4.98×10^{-5} μmols/min/mg of protein in contrast with that for benzonitrile as the substrate (13.4 μmols/min/mg). However, a double reciprocal plot (Fig. 1) gave a straight line with K_m = 2.94 mM. This apparent affinity was similar to that reported for benzonitrile (2.10 mM)[29]. These kinetic values were determined under the following conditions: substrate range, 1–5 mM (for the assay of amidase activity) or 0.5–6 mM (for the assay of nitrilase activity), pH 7.5, at 25°C. The amount of enzyme was 178 nmol (for the assay of amidase activity) or 0.54 nmol (for the assay of nitrilase activity) in a 3 ml reaction volume. Reaction time was 2 h (for the assay of amidase activity) or 10 min (for the assay of nitrilase activity).

When enzyme boiled for 5 min was used in the above reaction mixture, benzoic acid was not formed at all

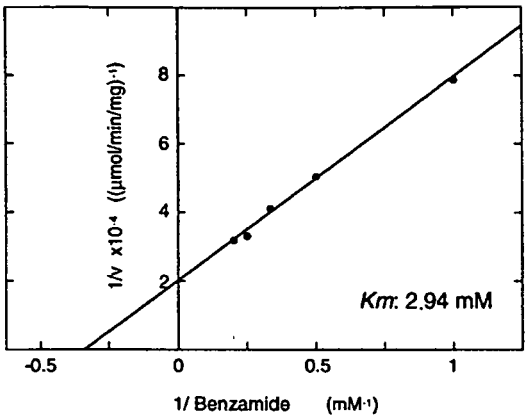

FIG. 1. Effects of substrate concentration on amidase activity by the nitrilase. The reaction was carried out in a 3 ml reaction mixture containing the nitrilase (178 nmol) and benzamide (1, 2, 3, 4 or 5 mM) in the buffer (pH 7.5).

from benzamide, demonstrating that the above amide degradation was not spontaneous. No benzoic acid was detected in the control experiment, in which the enzyme was not added to the reaction mixture containing benzamide as the substrate. We also performed the reaction using a blank sample as follows: according to the same procedure as used for the purification of the nitrilase from the *E. coli* transformant carrying pNJ20, cell-free extracts were prepared from the *E. coli* transformant carrying pUC19 (*i.e.*, the transformant which did not harbor the nitrilase gene) and fractionated with ammonium sulfate (25–50%, mass/vol), followed by dialysis against the 0.01 M buffer. The dialyzed solution derived from pUC19 did not exhibit amidase activity, while that derived from pNJ20 (which was prepared through the same procedure as used for the former dialyzed solution) showed amidase activity of 1.92×10^{-5} mmols/min/mg of protein. The ratio (= 0.575) of this value to that of the amidase activity of the purified nitrilase (3.34×10^{-5} μmols/min/mg) was similar to the ratio (= 0.590) of the nitrilase activity (9.03 μmols/min/mg) after ammonium sulfate fractionation relative to that of the purified nitrilase (15.3 μmols/min/mg). Together with the above high purity of the nitrilase used in these experiments, these findings demonstrated that amidase activity in the nitrilase was not due to impurities in either the enzyme or the substrate. This was also supported by the observation that the *R. rhodochrous* J1 mutant nitrilase, in which Cys165 (crucial for nitrilase activity) was replaced with alanine or serine, showed no amidase activity, as mentioned below.

We next investigated whether benzamide could also act as an inhibitor of nitrilase activity. The *R. rhodochrous* J1 nitrilase (0.27 nmol) was incubated at 25°C with 2, 2.5, 3.33, 5 or 10 mM benzonitrile in the presence of benzamide (0, 2.5, 5 and 7.5 mM) for 30 min (pH 7.5; 3 ml reaction volume), and then its activity was measured. Figure 2 shows the increasing inhibition with increasing amounts of benzamide. The inhibition constant, $K_{i(app)}$ (= apparent K_i), for benzamide with benzonitrile as the substrate was 16.6 mM, on the assumption that benzamide was a dead-end inhibitor, although it was surely a substrate for the nitrilase. The kinetic pattern was a competitive inhibition, suggesting competition for the same binding region by benzonitrile and benzamide at the enzyme active site cysteine residue. The apparent reversible nature of the inactivation process was evident, as follows: even after the nitrilase had been treated with benzamide (1, 6, and 40 mM)(resulting in remaining nitrilase activities of 95, 89, and 68%, respectively, in comparison with the non-treated enzyme), the resultant enzyme dialyzed against 0.01 M potassium phosphate buffer (pH 7.5) exhibited full nitrilase activity.

This is the first report of hydrolytic activity of nitrilase for amides. In the nitrilase reaction, nucleophilic attack on the nitrile carbon atom by the sulfhy-

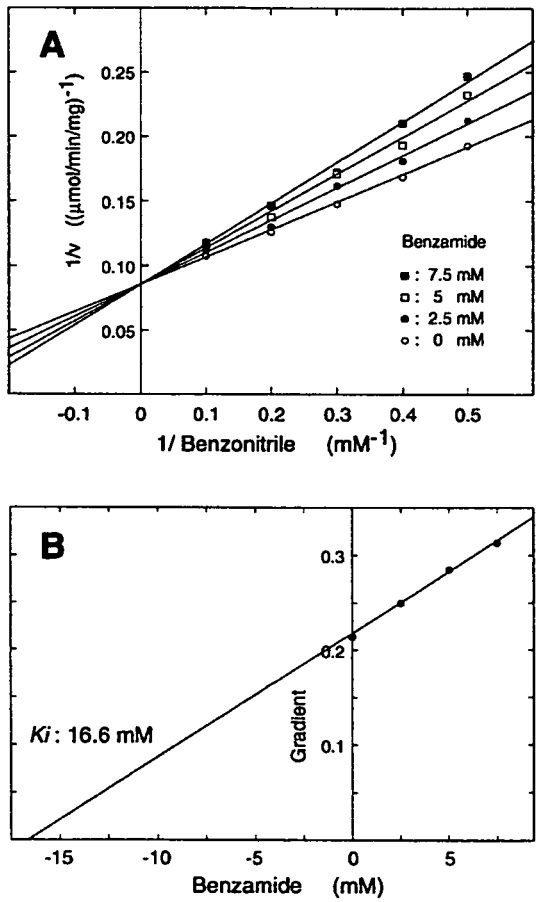

FIG. 2. Inhibition of the nitrilase reaction by benzamide. (A) Inhibition of the nitrilase reaction by benzamide; the *R. rhodochrous* J1 nitrilase was incubated with 2, 2.5, 3.33, 5 or 10 mM benzonitrile in the presence of benzamide (0, 2.5, 5 or 7.5 mM) for 30 min, and then its activity was measured. (B) A replot of the slope versus the millimolar concentration of benzamide.

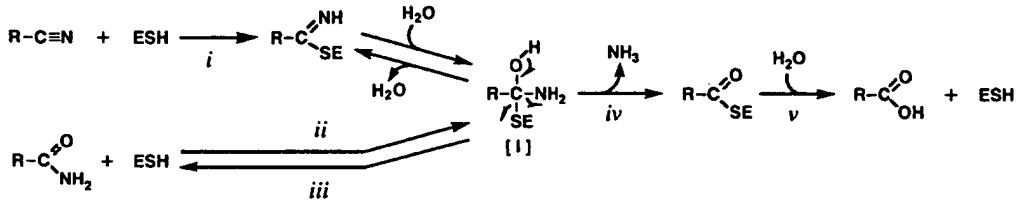

FIG. 3. Possible catalytic mechanism for the nitrilase reaction. The active sulfhydryl group is indicated by ESH. The tetrahedral intermediate is indicated by [I].

dryl group of the nitrilase (ESH in Fig. 3) leads to the formation of a tetrahedral intermediate ([I] in Fig. 3) via an enzyme-thioimidate (= enzyme-iminothiol ether) intermediate (route *i* in Fig. 3). Ammonium is then removed from the tetrahedral intermediate to yield an acyl-enzyme (route *iv*), followed by hydrolysis of the latter to an acid (route *v*). Particularly, the second step of the nitrilase reaction, in which attack by water on the covalently attached thioimidate requires activation of water to transform it into an adequate nucleophile, is unique to this enzyme, differing from the cysteine protease, papain [33].

Three nitrilases from *Pseudomonas* [34], *Fusarium* [35] and *Rhodococcus* ATCC39484 [36] have been shown to exhibit unique features in terms of their reaction products: they show approximately the same catalytic activity towards nitriles, but produce small amounts of amides (1–6% of the total products) as by-products [9]. These findings suggested that the tetrahedral intermediate could be broken down anomalously to produce an amide in place of the acid product (route *iii*) only when selected substrates are used. We would like to propose here a modified form of the possible reaction mechanism reported by both Mahadevan and Thimann [37], and Harper [38]. In this modified mechanism (Fig. 3), the reversible reaction (*i.e.*, routes *ii* and *iii*) could occur with nitrilases only under specified conditions. Route *v* cannot be reversible, as neither benzonitrile nor benzamide was formed from benzoic acid, when the latter's ammonium salt was used as the substrate even in the presence of a large amount of nitrilase (178 nmol of enzyme in a 3 ml reaction volume).

The *R. rhodochrous* J1 mutant nitrilase [24], in which Cys165 (crucial for nitrilase activity) was replaced with alanine or serine, did not show amidase activity, demonstrating that the catalytic sites responsible for nitrilase and amidase activities were the same. Therefore, the nitrilase-catalyzed degradation of benzamide would proceed through routes *ii*, *iv* and *v* at its unique sulfhydryl group, which plays an essential role in the function of the active site in the hydrolysis of nitriles.,

The C-N hydrolase superfamily comprised of nitrilase, cyanide hydratase, aliphatic amidase and β-ureidopropionase, all of which are physiologically important enzymes, has been proposed [25] based on the signature sequences, one of which contains the invariant cysteine identified as the active amino acid in our microbial nitrilases [2,9,21,24]. The former two enzymes act on the C-N triple bond of each substrate, but their reaction products are different: the nitrilase reaction product is an acid, in contrast with the cyanide hydratase reaction product which is an amide. Aliphatic amidase and β-ureidopropionase cleave the C-N single bond of each substrate to form the correponding acid; whereas the former enzyme acts on the $CO\text{-}NH_2$ (amide bond) of an amide, the latter acts on CO-NH- (which can be structurally called a peptide bond) of *N*-carbamoyl-β-alanine, and the resultant carbamate formed is further spontaneously degraded into carbon dioxide and ammonium. Cyanide hydratase converts the C-N triple bond of cyanide to an amide bond. In the reaction formula, cyanide hydratase is similar to nitrile hydratase [6,12,13,39,40](the family of which also includes thiocyanate hydrolase [41] as a member). However, there is no sequence similarity between these enzymes. Recently, Watanabe *et al.* [42] reported that cyanide dihydratase (= cyanidase) is also a member of the C-N hydrolase superfamily. The discovery of the nitrilase-catalyzed amidase activity supports the evolutionary relationship among the above members of the C-N hydrolase superfamily with differences in the substrate and product of each enzyme reaction.

Very recently, we found weak nitrilase activity of the amidase from *R. rhodochrous* J1 despite the observation that there is no sequence similarity between the amidase and nitrilase enzymes [43]. Although there is also no homology among the amidase, cysteine proteases and any enzyme in this C-N hydrolase superfamily, moreover, comparison of their reaction mechanisms could provide new insight for the construction of novel catalysts for the hydrolysis of amide bonds, peptide bonds and C-N triple bonds.

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